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Increased Virus Resistance in Transgenic Petunia with Heterologous *ZRNase II* gene

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Abstract

The production of transgenic petunia plants with heterologous *ZRNase II* gene and the evaluation of resistance to *Tobacco mosaic virus* in the resultant plants was the aim of our research.

We have generated transgenic petunia plants with *ZRNase* gene via *Agrobacterium*-mediated transformation. The presence of *ZRNase II* gene was confirmed by PCR-analysis. Orcin method revealed that RNase activity in transgenic petunia expressing *ZRNase II* gene was 1.34-2.10 fold higher compared to wild-type plants. RNase activity varied among different transgenic lines. Both petunia varieties used for production of transgenic lines differed in their natural resistance to *Tobacco mosaic virus.*The symptoms of the viral infection in the transgenic plants of the susceptible variety inoculated with the virus were less severe. Indirect ELISA confirmed 3.3 – 4.0 fold lower accumulation of viral antigenes in the infected transformed plants compared to the infected wild type ones.

Application of *ZRNase II* gene in creation of transgenic petunia leads to the generation of plants tolerant to *Tobacco mosaic virus*.

Keywords. *Genetically engineered plants, disease resistance, Tobacco mosaic virus, extracellular ribonucleases.*

1. Introduction

Petunia is an economically important ornamental plant, which was traditionally propagated by seeds. The emergence of modern hybrid cultivars called 'Surfinia' led to the increasing of vegetative propagation on petunia market. Introduction of vegetatively propagated *Petunia* hybrids led to an increased risk of virus infections of propagated material (Chung *et al.*, 2007). Nowadays, 26 RNA containing viruses and 4 viroids were identified to infect *Petunia* plants (Sastry*et al*., 2019). Among them the most detrimental are *Tobacco mosaic virus* (TMV), *Petunia vein-clearing virus, Tomato mosaic virus, Potato virus Y, Alfalfa mosaic virus, Cucumber mosaic virus*, and *Broad bean wilt* I *virus.* They are often identified in mixed infections (Lasemann, 1996; Chung *et al*., 2007). Viral infections decrease decorative characteristics of cultivars, which become the source of the virus for uninfected plants.

Except of its direct economic value as an ornamental plant, petunia is attractive as a model system. There is a need in modern plant biology for the model systems (beyond *Arabidopsis)* for different specialized demands. Such advantages of petunia as short generation time, easy cultivation, efficient protocol for genetic transformation, large leaves and flowers suitable for a number of analysis, make it a convenient model for investigations (Vandenbussche *et al*., 2016). Petunia can also be used for testing of various antiviral strategies prior to testing them on recalcitrant species.

Transgenic virus resistant plants can be produced by approaches of modern biotechnology. Antisense RNAs, coat protein mediated resistance, microRNAs, hammerhead ribozyme, plantibodies, replicase mediated resistance, ribosome-inactivating proteins (RIP), RNA dependent RNA polymerase mediated resistance, RNA interference, RNA satellites, and some ribonucleases have been tested as a putative antiviral strategies previously (Sudarshana *et al*., 2007; Galvez *et al*, 2014). Sometimes viruses have the capability to supress plant defense against virus infections (Abdelkhalek and Sanan-Mishra, 2018), so the more antiviral strategies we have, the better is plant disease control.

ZRNase II gene was isolated from *Zinnia elegance.* The gene encodes an extracellular ribonuclease, which hydrolyzes viral RNAs during their penetration into the plant cell (Kochetov and Shumny, 2017). The enzyme digests viral RNAs, thus localizing the infection, retarding and mitigating the severity of infection symptoms in tobacco (Sangaev *et al*., 2007, Trifonova *et al*., 2012) and potato (Potrochov *et al*., 2021).

Production of transgenic petunia plants expressing heterologous extracellular ribonuclease of *Zinnia* and evaluation of virus resistance in obtained plants was the aim of our research.

2. Materials and methods

2.1. Plant material

Petunia cultivars were originated from a pack of mixed commercial seeds and chosen based on floral phenotype. These cultivars, shown in Figure 1, are currently being maintained vegetatively for research purposes in the *in vitro* collection at the Institute of Cell Biology and Genetic Engineering. The varieties with the highest regeneration capacity from leaf explants (M1 and P5) were chosen for *Agrobacterium*-mediated genetic transformation (data not shown)**.** Plants are available upon request.

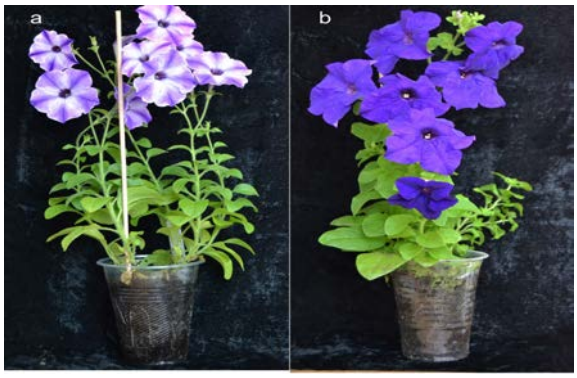

Figure 1. General appearance of varieties used for genetic transformation. M1 (a) and P5 (b) petunia cultivars.

2.2. Agrobacterium-mediated transformation

Transgenic petunia plants were produced according to Lutke (2006). Petunia leaf discs were transformed with *A. tumefaciens* AGL0 strain harboring pbi-RNS vector. T-DNA of pBi-RNS vector included *ZRNase II* gene of S-like RNase of *Zinnia elegans* (817 bp, Gene Bank accession number U19923.1) controlled by p35S CaMV, derived from the cauliflower mosaic virus (CaMV) and neomycin phosphotransferase gene *(npt II)*, under control of nopaline synthase promoter (pNOS), as shown in Figure 2 (Sangaev *et al*., 2007). Plants were regenerated and selected on regeneration MS medium (Murashige and Skoog, 1962) with 1 mg/l of BA, 0.1 mg/l of NAA, 100 mg/l kanamycin and 400 mg/l cefotaxime.

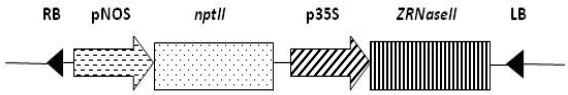

Figure 2. Schematic representation of the T-DNA region of the plasmid pBi-RNS used for petunia transformation. LB, left border; pNOS, nopaline synthase promoter; *npt II*, neomycin phosphotransferase gene; 35S, promoter, derived from the cauliflower mosaic virus (CaMV); *ZRNase II*, S-like RNase gene of *Zinnia elegans*; RB, right border.

2.3. Polymerase chain reaction assays (PCR and RT-PCR)

PCR was used to analyze the presence of gene of interest and selective gene in kanamycin resistant plants.

DNA was isolated from putative transgenic plants, positive and negative controls using a standard kit «NeoPrep100 DNA plant» («Neogene», Ukraine) according to the manufacturer's instructions. The transformation events were confirmed by standard PCR techniques (Sambrook *et al*., 1989) using «PCR MIX 2-R» kit («Neogene», Ukraine). The primers and expected size of the PCR fragments are shown in the Table 1. The amplification of *ZRNase II* gene fragment was carried out under following conditions: 3 min at 94ºC, 42 cycles (30 s at 94ºC; 30 s at 55ºC; 45 s at 72ºC), 10 min at 72ºC. The amplification of *npt II* gene fragment was carried on under following conditions: 4 min at 94ºC, 8 cycles (30 s at 94ºC; 45 s at 68ºC; 30 s at 72ºC), 25 cycles (30 s at 94ºC; 30 s at 60ºC; 30 s at 72ºC), 1 min at 72ºC. The amplification conditions for *virC* fragment were: 3 min at 94ºC, 40 cycles (30 s at 94ºC; 47 s at 55ºC; 30 s at 72ºC), 5 min at 72ºC. The samples were fractionated in 1% agarose gel in LB (lithium borate) buffer (Brody *et al*., 2004).Thermo Scientific GeneRuler 1 kb (250 –10000) bp was used as a molecular-weight size marker.

Table 1. The primers used for PCR analyses and expected size of amplified fragments.

Gene	Primers	Annealing temperature, ºC	Amplified fragment, bp
ZRNase II	5'-GAATCTAGAAATTTAGAATGAAGGA-3'	55	720 (Sangaev *et al*., 2007)
	5'-ACACTCGAGCACACAAACATGAAGA-3'		
npt II	5'-GAG GCT ATT CGG CTA TGA CTG-3'	68, 60	700 (Bińka, 2011)
	5'-ATC GGG AGC GGC GAT ACC GTA-3'		
virC	5'-ATC ATT TGT AGC GAC T-3'	55	720 (Sawada *et al*., 1995)
	5'-AGC TCA AAC CTG CTT C-3'		

The transgene expression in petunia plants was examined by RT-PCR analysis. Total RNA was isolated with "RNA isolation kit" ("Zymo Research"). "First strand cDNA synthesis kit" ("Fermentas") was used for cDNA generation according to manufactures manual. Each sample was analyzed with and without addition of reverse transcriptase M-MuLV. The amplification was conducted as described above with the same primers for *ZRNase II*. The amplification products were separated on 1.2% agarose gel in LB buffer.

2.4. Conditions for growing plants in greenhouse and inoculating with virus

Wild type and transgenic (2 of M1 and 3 of P5) lines of petunia were propagated *in vitro* via stem cuttings on MS medium. Rooted plants were acclimated to the greenhouse condition and grown at 24ºC under a photoperiod of 16h light and 8 h darkness.

One month after transfer to soil, transgenic and wild type petunia plants were inoculated with 100 µl of TMV in inoculation buffer (the concentration of purified powder-dried virions in 0.05 M sodium phosphate buffer, pH 7.0 was 250 µg/ml). Tomato plants with severe symptoms of viral infection were the source of TMV sample. Upper

fully expanded leaves of experimental petunia plants were dusted with carborundum and inoculated by rubbing with the TMV suspension. Mock-inoculated plants were inoculated with the buffer without virus. After inoculation, plants were rinsed with tap water and incubated in the greenhouse (20 ± 5ºC).

Each experiment involving TMV inoculations was performed in six replicates (a single plant represented one replicate). Afterwards, plants were monitored for the development of viral symptoms.

2.5. RNase activity detection

RNase activity was determined by colorimetric method according to Jain *et al*. (2020).

2.6. Enzyme linked immunosorbent assay (ELISA).

Accumulation of viral antigens was analyzed by indirect ELISA. Primary polyclonal rabbit antibodies to TMV and secondary goat anti-rabbit antibodies (Sigma) were used according to the manufacturer's instructions and Crowther (1995). Upper leaves from six distinct plants per each variant of experiment were drilled, and the probes were tested by ELISA. Samples were considered positive according to condition mentioned in Arli-Sökmen *et al*. (1998).

2.7. Statistical evaluation

Analysis of variance was used to calculate the least significant differences by Statistica 5.5 and MS Excel 2016 software. Data were compared by Mann-Whitney U test in order to evaluate statistical differences between each transgenic line and corresponding non-transgenic controls. Each transgenic line and non-transgenic controls were represented by six plants. Experiments were repeated triple.

3. Results

3.1. Selection of the putative transgenic plants.

Shoots began to regenerate from leaf disks in a month after *Agrobacterium* treatment on regeneration MS medium supplemented with kanamycin sulfate, as shown in Figure 3. Kanamycin-resistant shoots of petunia were rooted spontaneously on MS medium with 100 mg/l of kanamycin sulfate. Only the most vigorous and rooted lines were selected from each variety. Eight putative transgenic lines with pBi-RNS vector of M1 petunia and 10 lines of P5 were analyzed further.

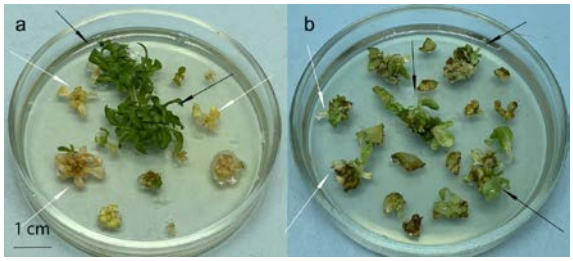

Figure 3.Genetic transformation of *Petunia* using *Agrobacterium tumefaciens* AGL0 with pBi-RNS vector (bar 1 cm). Regeneration of M1 (a) and P5 (b) kanamycin resistant shoots on MS regeneration medium containing100 mg/l of kanamycin sulfate 8 weeks after cocultivation. Black arrows indicate resistant shoots while white arrows mark the susceptible ones.

3.2. PCR analyses of transgenic plants

Putative transgenic plants were analysed with primers specific to *npt II* and *ZRNase II* genes. Non-transformed M1 and P5 petunia cultivars were PCR tested to confirm the absence of the transgenes. There was no amplification of the tested fragments with primers to both genes in non-transformed plants. All selected kanamycin resistant lines demonstrated the presence of amplified fragments with primers to *npt II* (data not shown) and *ZRNase II* genes as shown in Figure 4. Fragment of *virC* gene, characteristic to *A.tumefaciens* was not found. RT-PCR revealed the expression of gene of interest (Figure 5).

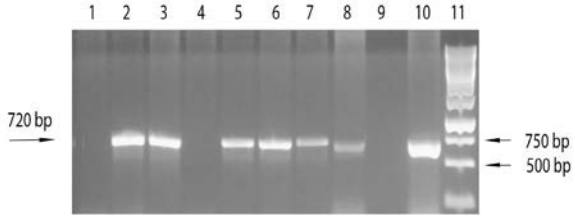

Figure 4. Polimerase chain reaction analysis of transgenic petunia for the *ZRNase II* gene (720 bp). Lanes: 1– M1 non-transgenic plant*;* 2, 3 – transgenic lines M1T1, M1T2; 4 – P5 non-transgenic plant*;* 5-8 – transgenic lines P5T1, P5T2, P5T3; 9 – negative control, without DNA; 10 – positive control, total DNA of *A. tumefaciens* AGL0 with pBi-RNS plasmid; 11 – Thermo Scientific GeneRuler 1 kb, 250 –10000 bp.

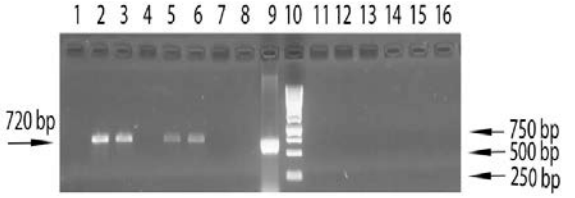

Figure 5. RT-PCR with primers for the *ZRNase II* gene (720 bp) in samples from petunia leaves. Amplification with cDNA (lanes 1–6) and appropriate RNA samples (11 – 16) isolated from petunia. Lanes: 1, 11 – cultivar M1, wild type; 2, 12 – transgenic line M1T1; 3, 13 –transgenic line M1T2; 4, 14 – cultivar P5, wild type; 5, 15 – transgenic line P5T1; 6, 16 –transgenic line P5T2; 7 – reagent mix without cDNA addition; 8 – reagent mix without RNA addition; 9 – total DNA of *A. tumefaciens* AGL0 with pBi-RNS plasmid; 10 – DNA marker ladder (Thermo Scientific GeneRuler 1 kb, 250 –10000 bp).

For further tests on RNase activity and virus resistance, M1T1, M1T2 transgenic petunia lines of M1 variety and P5T1, P5T2, P5T3 lines of P5 expressing S-like RNase of *Zinnia elegans* were selected. These lines were propagated *in vitro* and transferred to soil.

3.3. RNase activity assay

RNase activity of the transgenic plants significantly ($P \leq 0{,}05$) exceeded that of non-transgenic ones as shown in Figure 6. Total RNase activity of different transgenic lines of P5 variety exceeded from 1.83 to 2.10 fold the activity of wild type line. The RNase activity in M1 based lines was from 1.34 to 1.85 fold higher than in appropriate wild type variety.

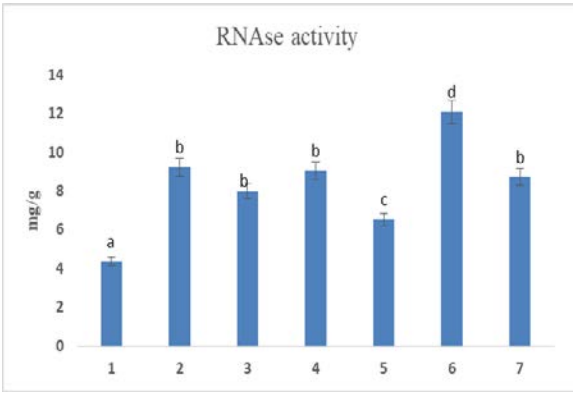

Figure 6. The RNase activity in petunia leaf extracts. The amount of destroyed RNA in mg per g of plant tissue (fresh weight) was estimated. 1, 5 – non-transformed 5P and M1 petunia plants, respectively; 2-4 – P5T1, P5T2, P5T3 transgenic lines of 5P petunia; 6-7 – M1T1 and M1T2, transgenic lines of M1 petunia. The same letters mark bars without significant differences. ($P \leq 0.05$).

3.4. Infection of petunia plants with TMV.

After mechanical inoculation of fully expanded leaves of petunia with TMV inoculum, the systemic signs of viral infection were evaluated. The typical symptoms of TMV infection such as mosaic on leaves, mottling, rugosity of leaf blades appeared on the upper leaves in wild type petunia plants on the third week after inoculation, as shown in Figure 7. The upper leaves of infected non-transgenic M1 plants manifested severe infection symptoms in contrast to the wild type P5 petunia. The latter showed only slight rugosity of leaves. Leaf morphology of infected transgenic cultivars (M1 (Figure 7a, c, e) and P5 varieties) and plants inoculated with buffer was similar.

Figure 7. Systemic signs of infection in TMV inoculated non-transformed and transformed petunia plants expressing S-like RNase of *Zinnia elegans.* Detached petunia leaves (a – c): a – M1 line mock-infected, b – M1 line inoculated with TMV, c– transgenic M1T1 line inoculated with TMV. Development of visual systemic symptoms of viral infection on TMV infected control non-transformed M1 petunia plants (d) versus the transgenic M1T1 petunia plants *(e)*.

3.5. Detection of TMV in plants by ELISA

Systemic TMV infection was investigated in the upper fully expanded young leaves of intact and infected plants by ELISA. The results are presented in Figure 8. Viral antigens were recovered from the upper non-inoculated leaves of infected M1 wild type plants indicating the systemic viral movement. In non-transgenic M1 variety the difference in extinction values was almost 4-fold between mock and infected plants. According to Arli-Sökmen *et al*. (1998) viral particles are considered to be accumulated in plants if there is at least 2-fold difference between the absorbance values (A 405) of infected and virus-free samples. In contrast, in the P5 variety (where minor signs of viral infection were observed) the difference was less obvious (1.62 folds). There was the significant difference in the antigen amount between wild type P5 and M1 varieties. Antigen accumulation was higher in M1 than in P5. The absorbance values in samples of infected M1 variety 2.6 fold exceeded those of infected P5. The infected transgenic plants of M1 variety manifested much lower ELISA values than the infected wild type plants (from 3.3 to 4.0 fold). Extinction values of infected P5 and M1 transgenic varieties, compared to mock ones, were lower 1.15-1.32 and 0.98-1.19 fold respectively. These means did not exceeded those of the virus-free samples by at least a factor of two (Arli-Sökmen *et al*., 1998). So, they can be considered undistinguishable from mock treated plants. We can consider that there was no significant accumulation of viral particles in transgenic plants.

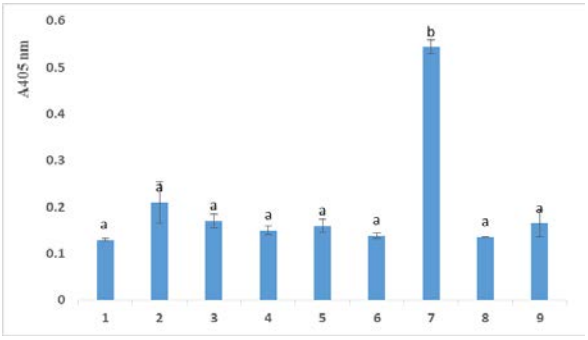

Figure 8. Indirect ELISA extinction values of different petunia lines. 1, 6 – inoculated with buffer non-transformed control P5 and M1 petunia plants, respectively; 2, 7 – inoculated with TMV non-transformed control P5 and M1 petunia plants, respectively; 3-5 – inoculated with TMV transformed lines of P5 petunia - P5T1, P5T2, P5T3; 8-9 – transformed lines of M1 petunia M1T1, M1T2. Samples were considered to be positive when the A 405 absorbance values exceeded the mean of the virus-free samples by at least a factor of two. The same letters mark bars without significant differences.

4. Discussion

Nowadays, there is an insistent demand for efficient control of pathogens injuring important cultivated species. RNases are involved in the wound response and induction of the defense-related genes. That is one of the ways in which plants respond to mechanical injury and prevent viral infection (LeBrasseur *et al*., 2002). The effect of heterologous *ZRNase II* gene expression on TMV resistance of transgenic petunia plants was investigated. It is important to note that the transgene expression had no influence on plant morphology in the transgenic petunia

plants grown in the greenhouse. No obvious effect of this gene on transgenic petunia plant growth and development was observed in accordance to similar previous research on tobacco and potato (Sangaev *et al*., 2007; Potrochov *et al*., 2021). The absence of negative influence of excessive RNases on transgenic plants can be associated with subcellular compartmentalization of the plant RNases outside the cell (Hugot *et al*., 2002)

In our experiments, total RNase activity of wild type lines (M1 and P5) differed, due to their genetic background. However, in both cases the RNase activity of transgenic lines, containing the extracellular *ZRNase II* gene, were higher. There were differences also among the transgenic *ZRNase II* petunia lines. The transgenic plants had total RNase activity higher 1.34 – 2.10 fold compared to the control. Gene position effect and the levels of expression could explain the differences in activity. Previously, tobacco plants, which were transformed with a vector containing *ZRNase II* gene, were characterized with RNase activity 1.6 – 3.0 and 3.5 – 14.4 fold higher than in the control, presumably depending on regulatory elements (Sangaev *et al*., 2007; Trifonova *et al*., 2012). Transgenic potato plants transformed with pBi-RNS vector manifested total activities of RNases 1.2 times higher as compared to non-transgenic plants (Potrochov *et al*., 2021). Although the activity of total RNases increased moderately, the transgenic plants tolerated inoculation with the high viral doses, in contrast to non-transgenic ones. This is in accordance with the fact that PR-4 protein, the product of L3 gene from *Capsicum chinense*, with both RNase and DNase activity, protected plants of this species from the most of tobamoviruses, despite the very low contribution to the bulk activity of nucleases in infected plants (Guevara-Morato *et al*., 2010).

We found that wild type P5 and M1 lines differed in their tolerance to TMV. The visual signs of infection after viral inoculation both with subsequent ELISA demonstrated different susceptibility of wild type M1 and P5 petunia plants to TMV infection, as shown in Figure 8. On the third week after inoculation, we observed 4-fold rise of viral antigens for infected wild type M1 line and no significant increase for P5 line, as compared to the results for mock infected plants. Symptoms of tobamoviruses in naturally infected petunias usually begin to appear from the second week after inoculation and differ among cultivars (Cohen *et al*., 1999). In the infected plants they can range from the complete absence of detectable symptoms to different severity forms of mottling and mosaic, leaf deformations. Infection can also reduce the number of flowers along with size and cause the break of flower colour. Cohen *et al*. (1999) observed no viral signs developed on the upper leaves of a number of cultivars up to four weeks post inoculation with TMV, but detection of the viral particles from non-inoculated leaves revealed their systemic movement even in symptomless plants. We can consider that P5 line has high natural tolerance to TMV infection for its minor symptoms and low extinction values of ELISA. In contrast, M1 is a susceptible cultivar. The reason of the various susceptibility to virus among the varieties may be due to difference in the genetic background. The difference among the petunias in tolerance to TMV is consistent with the previous findings (Cohen *et al*., 1999).

The aim of our study was looking at systemic responses to TMV infection in petunia plants. The inoculation dose of 250 μg/ml was used in our experiment in order to obtain the systemic infection. Although we used such high viral concentration for petunia inoculation, the absorbance value in transgenic plants was similar to the mock infected plants. The difference in the observed visual symptoms and antigen accumulation retained after three weeks post inoculation with TMV between wild type plants and two transgenic clones of susceptible petunia variety M1. Absorbance values for infected transgenic P5 clones were similar to those inoculated with buffer. The absorbance values for infected wild type P5 plants (control) exceeded those in transgenic P5 clones, but were not substantial on the third week post inoculation. Previously, systemic responses were observed in experiments when 12.5 times less concentrated TMV inoculum dose with 20 μg/ml of TMV for experimental infection of wild type petunias and 25 times lower viral concentration for inoculation of tobaccos were used (Cohen *et al*., 1999; Trifonova *et al*., 2012). It was shown earlier that the response of transgenic tobacco plants (with *ZRNase II* gene) infected with TMV depended on the concentration of virus (Trifonova *et al*., 2012). These researchers observed that in the case of low (0.01μg/ml) or medium (0.1 μg/ml) content of the viral particles in the inoculum, the virus accumulation and development of infection symptoms were absent or delayed in transformed plants as compared to the control ones. If the TMV concentration in inoculum was 10 μg/ml, the differences between infected control and transformed plants were less obvious at 3 weeks after inoculation) (Trifonova *et al*., 2012). Thus, our results demonstrate the easier infection course in inoculated transgenic plants that corroborates previous findings (Sangaev *et al*., 2007; Trifonova *et al*., 2012, Potrochov *et al*., 2021).

Heterologous recombinant RNases were demonstrated to increase antiviral resistance in transgenic plants previously (Ishida *et al*., 2002). However, there is a report that the protection effectiveness of some proteins with RNase activity can be virus specific (Guevara-Morato *et al*., 2010). Some reports also demonstrate the direct protection of plant RNases against fungal pathogens in the apoplastic compartment (Hugot *et al*., 2002). Thus, the transgenic *ZRNase II* plants present an interesting model for further investigation of the plant resistance to a number of viruses and fungi.

5. Conclusions

Transgenic petunia plants were transformed with heterologous *ZRNase II* gene. The transgenic plants manifested increased levels of RNase activity and the reduction in virus antigen accumulation in susceptible to *Tobacco mosaic virus* cultivar. Genetic transformation of this ornamental species with extracellular ribonuclease gene resulted in production of plants tolerant to TMV.

6. Data Availability Statement

The data that support the findings of this study are available from the corresponding author, OO, upon reasonable request.

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